

Full Length Research Paper

# Antimelanogenic and antioxidant activities of *Bifidobacterium infantis*

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The study was aimed at investigating the antimelanogenic and antioxidative effects of *Bifidobacterium infantis* culture filtrate. The efficacy of the bacterial culture filtrate was evaluated spectrophotometrically. The results revealed that *B. infantis* culture filtrate effectively scavenge 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ABTS radicals, and show potent reducing power in a dose-dependent pattern. Additionally, the bacterial culture filtrate suppresses murine B16F10 tyrosinase activity and the amount of melanin in a dose-dependent manner. We conclude that the *B. infantis* culture filtrate may be applied in the formulations of skin care cosmetics.

**Key words:** *Bifidobacterium infantis*, tyrosinase, melanin, antioxidant.

## INTRODUCTION

Melanin plays an important role in protection the skin against ultraviolet light (UV) light injury and is responsible for skin color. It is reported that overproduction and accumulation of melanin result in several skin disorders such as melasma, age spots, other hyperpigmentation syndrome and freckles (Briganti et al., 2003). Tyrosinase is the rate-limiting enzyme in melanin biosynthesis pathway (Seo et al., 2003). It has been evidenced that other enzymes such as tyrosinase-related protein-1

(TRP-1) and tyrosinase-related protein-2 (TRP-2) also contribute to the production of melanin (Hearing and Jimenez, 1987; Tsukamoto et al., 1992; Jimenez-Cervantes et al., 1994). Recently, various inhibitors of melanogenesis have been applied in skin care products for the prevention of hyperpigmentation (Funasaka et al., 2000). Active oxygen species and free radicals have been recognized as important factors in the pathogenesis of human diseases such as atherosclerosis, aging and cancer (Halliwell et al., 1992). It is reported that UV exposure generates reactive oxygen species (ROS) and excessive ROS are associated with several skin disorders (Yasui and Sakurai, 2003). Additionally, it is reported that melanogenesis produce hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and other ROS which makes the melanocytes under low-grade oxidative stress. It is evidenced that ROS scavengers and inhibitors of ROS generation may down-regulate melanogenesis-induced by UV exposure (Yamakoshi et al., 2003).

Therefore, antioxidants such as ascorbic derivatives and reduced glutathione (GSH) have been applied as

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**Abbreviations:** DPPH, 2,2-Diphenyl-1-picryl-hydrazyl; ABTS, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); TRP-1, tyrosinase-related protein-1; TRP-2, tyrosinase-related protein-2; UV, ultraviolet; ROS, reactive oxygen species; GSH, glutathione; BCRC, bioresource collection and research center; MRS, man rogosa sharp; MC1R, melanocortin 1 receptor; BHA, butylated hydroxyanisole

inhibitory agents of melanogenesis (Imokawa, 1989; Kumano et al., 1998). *Bifidobacteria* are gram-positive obligate anaerobes and considered to promote the health of host intestinal tract because of their beneficial effects including reduction of harmful bacteria and toxic compounds, regulation of the state of the intestine, modulation of immune response and anticancer activity (Gibson and Wang, 1994; Hooper and Gordon, 2001; Femia et al., 2002; Ouwehand et al., 2002). Besides, it is reported that orally administered *Bifidobacteria* species can improve nutritional value of food and control intestinal infections (Gilliland, 1990). Recently, numerous human clinical trials suggested that probiotic supplementation might be useful in the treatment of atopic dermatitis (Kalliomaki et al., 2001; Rautava and Isolauri, 2002; Kalliomaki et al., 2003; Kalliomaki et al., 2007; Puch et al., 2008). Moreover, some reports have demonstrated that certain bacterial extracts including *Lactobacillus johnsonii*, *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus acidophilus*, have anti-microbial and anti-adhesion properties when applied to cutaneous and mucous surfaces (Ouwehand et al., 2003; Rodriguez et al., 2005). Furthermore, *B. infantis* has been reported to show several therapeutic effects such as prevention of interferon-gamma-induced keratinocyte apoptosis, anti-inflammation activity and protective effects for colitis (Cinque et al., 2006; Tanabe et al., 2008; Van der Kleij et al., 2008). However, there is no report about application of *B. infantis* culture filtrate in cosmetics for potential skin care use. In the present study, we attempted to investigate the effects of *B. infantis* culture filtrate on the mushroom tyrosinase activity, intracellular tyrosinase activity and melanin content in B16F10 murine melanoma cells. Moreover, we looked into the inhibitory effect of *B. infantis* culture filtrate on melanogenesis associated with its antioxidant properties by assessing its antioxidant and free radical scavenging activities.

## MATERIALS AND METHODS

### Bacterial culture

The *B. infantis* strain (BCRC 14602) was obtained from the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Hsinchu, Taiwan. The bacterial strain was cultivated in Man Rogosa Sharp (MRS) broth (Difco, Detroit, MI and USA) supplemented with 0.4g / l cysteine-HCl (Sigma Chemical Co. St. Louis, USA) anaerobically overnight at 37°C. Before experimental use, bacterial strain was subcultured twice in cysteine-containing MRS broth. After that, 1% (v/v) of each culture was transferred to a 50 ml cysteine-containing MRS broth and incubated anaerobically at 37°C for 48 h in an anaerobic incubation system (ThermoForma, Model 1025, Marietta, USA) that was continuously sparged with a mixture of 10% carbon dioxide, 10% hydrogen and 80% nitrogen (Toyogas, Taichung and Taiwan). After incubation, bacterial cells were removed by centrifugation (10,000 g / 10 min / 4°C) and supernatant was passed through a cellulose acetate filter (0.45 µm) to get bacterial culture filtrate.

## Determination of antimelanogenic activity

### Anti-mushroom tyrosinase assay

In order to assay the potential inhibitory effects of *B. infantis* culture filtrate on mushroom tyrosinase, dose-dependent inhibition experiments were carried out in triplicate as described previously with a minor modification (Bilodeau et al., 2001). Briefly, 10 µl of aqueous solution of mushroom tyrosinase (1000 units) was added to a 96 well microplate, in a total volume of 200 µl mixture containing 5 mM L-dopa dissolved in 50 mM phosphate buffer (pH 6.8). The assay mixture was incubated at 37°C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined by spectrophotometric analysis at 490 nm absorbance.

### Intracellular melanin content measurement

B16F10 melanoma cells (ATCC CRL-6475; BCRC60031) were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco, NY and USA) and penicillin/ streptomycin (100 IU/50 µg / ml) in a humidified atmosphere containing 5% CO<sub>2</sub> in air at 37°C. Intracellular melanin content was measured as described by Tsuboi et al. (1998) with some modifications (Tsuboi et al., 1998). The cells were treated with -MSH (100 nM) for 24 h and further treated with either *B. infantis* culture filtrate (final concentration 1.0, 2.5, 5.0, 7.5 % v/v) or kojic acid (200 µM) or arbutin (1 mM) for another 24 h. After treatments, the cells were detached by incubation in trypsin/EDTA and subsequently centrifuged at 5,000 g for 5 min, and then the cell pellets were solubilized in 1 N NaOH at 60°C for 60 min. The melanin content was assayed by spectrophotometric analysis at 405 nm absorbance.

### Intracellular tyrosinase activity assay

B16F10 intracellular tyrosinase activity was determined as described previously with slight modifications (Yeh Yang et al., 2006). Briefly, the cells were treated with α-MSH (100 nM) for 24 h and then further treated with various concentrations of *B. infantis* culture filtrate (final concentration 1.0, 2.5, 5.0, 7.5% v/v) or kojic acid (200 µM) or arbutin (1 mM) for another 24 h. After treatments, the cells were washed twice with phosphate-buffered saline and homogenized with 50 mM PBS (pH 7.5) buffer containing 1.0% Triton X-100 and 0.1 mM PMSF. Cellular extracts (100 µl) were mixed with freshly prepared L-Dopa solution (0.1% in phosphate-buffered saline) and incubated at 37°C for 30 min. The absorbance at 490 nm was measured with a microplate reader Gen 5™ (BIO-TEK Instrument, USA) to monitor the production of dopachrome.

## Determination of antioxidant activity

### DPPH scavenging activity assay

The antioxidant activity of *B. infantis* culture filtrate was first determined by measuring the DPPH scavenging ability (Brand-Williams et al., 1995) as modified by Sanchez-Moreno et al. (1998). Bacterial culture filtrates at various concentrations (final concentration 2.5, 5.0, 7.5% v/v) were added to 2.9 ml of DPPH (60 µM) solution. When DPPH reacts with an antioxidant that can donate hydrogen, it gets reduced form and the resulting decrease in absorbance at 517 nm was recorded using a UV-Vis spectrophotometer (Jasco, V-630, UV-Vis spectrophotometer). In this study, vitamin C (50 µM) and vitamin E (50 µM) were used as antioxidant standards.

### **ABTS<sup>+</sup> scavenging capacity assay**

The ABTS decolorisation assays were carried out as previously described (Re et al. 1999) and it involves the generation of ABTS<sup>+</sup> chromophore by oxidation of ABTS with potassium persulfate. The ABTS radical cation (ABTS<sup>+</sup>) was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark for at least 6 h before use. Absorbance at 734 nm was measured 10 min after mixing of different concentrations of the *B. infantis* culture filtrate (final concentration 2.5, 5.0, 7.5% v/v) with 1 ml of ABTS<sup>+</sup> solution. The ABTS<sup>+</sup> scavenging capacity of the filtrate was compared with that of vitamin C (60 μM) and vitamin E (60 μM).

### **Determination of reducing power**

The reducing power of the extract was determined according to the method of Oyaizu (1986). Different concentrations of the bacterial filtrate or vitamin C (44 μM) or vitamin E (44 μM) was mixed with phosphate buffer (2.5 ml, 0.2 M and pH 6.6) and potassium ferricyanide [K<sub>3</sub> Fe (CN)<sub>6</sub>] (2.5 ml, 1% w/v). The mixture was incubated at 50° C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10% w/v) was added to the mixture, which was then centrifuged at 1000 g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl<sub>3</sub> (0.5 ml, 0.1% w/v) and the absorbance was measured at 700 nm in a UV-Vis spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power.

### **Statistical analysis**

Statistical analysis of the experimental data points was performed by the ANOVA test, which was used for comparison of measured data using SPSS 12.0 statistical software (SPSS INC. Chicago, USA). Differences were considered as statistically significant at  $p < 0.05$ .

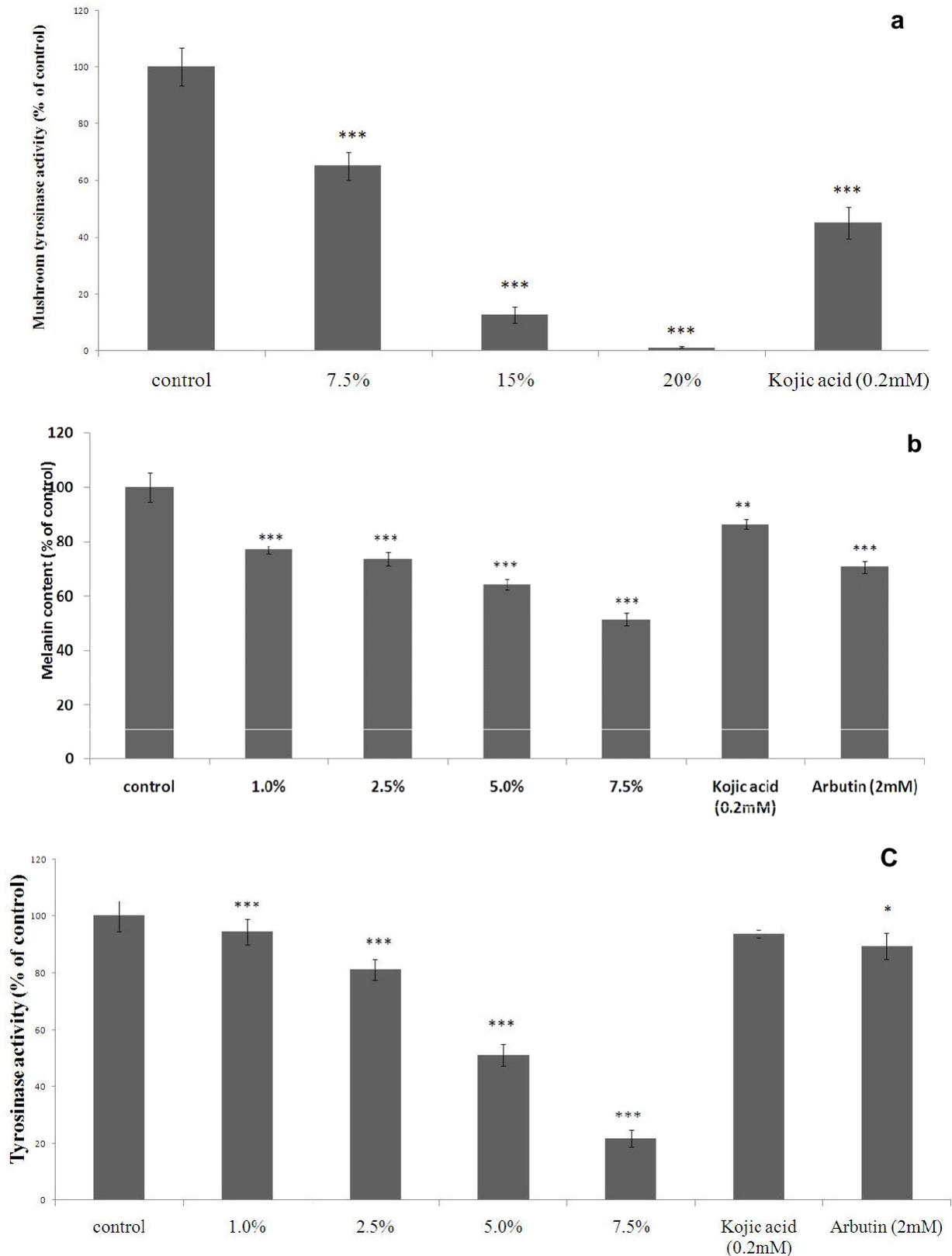
## **RESULTS AND DISCUSSION**

To determine the potential whitening activity of *B. infantis* culture filtrate, the potential inhibitory effects of the bacterial culture filtrate on mushroom tyrosinase and B16F10 intracellular tyrosinase activities were assayed. Furthermore, the melanin content in B16F10 cells was also measured after treatment with the culture filtrate or positive standards. Figure 1A indicated that the mushroom tyrosinase activity was inhibited by different concentrations of *B. infantis* culture filtrate in a dose-dependent manner. The remaining tyrosinase activity was  $65.2 \pm 4.8$ ,  $12.7 \pm 2.9$  and  $1.2 \pm 0.25\%$  for 7.5, 15 and 20% of bacterial culture filtrate, respectively (Figure 1A). Meanwhile, the remaining tyrosinase activity was  $45.2 \pm 5.5\%$  for kojic acid. However, lower concentrations of the bacterial filtrate (1.0, 2.5, 5.0% v/v) did not show apparent tyrosinase inhibitory activity (data not shown). To determine the effect of *B. infantis* culture filtrate on melanogenesis, B16F10 melanoma cells were stimulated with 100 nM  $\mu$ -MSH for 24 h and cultured in the presence of *B. infantis* culture filtrate (1.0, 2.5, 5.0 and 7.5% v/v), kojic acid (200 μM) or arbutin (2 mM) for another 24 h.

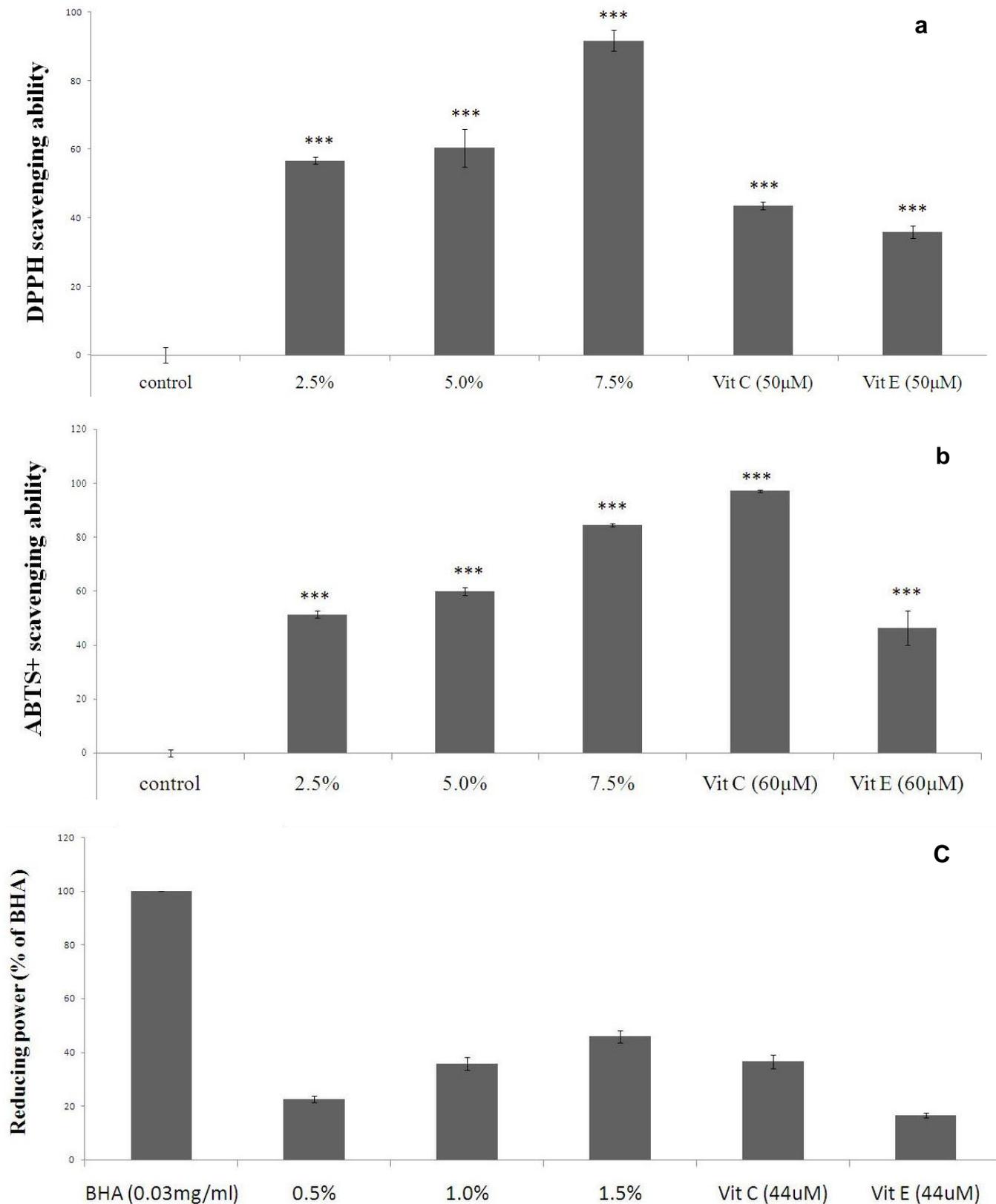
After treatment, the melanin content was  $77.1 \pm 1.5$ ,  $73.5 \pm 2.6$ ,  $64.3 \pm 2.1$ ,  $51.5 \pm 2.3\%$  for 1.0, 2.5, 5.0 and 7.5% of bacterial culture filtrate, respectively (Figure 1B).

Meanwhile, the melanin content was  $86.4\% \pm 1.8\%$  for kojic acid and  $70.6 \pm 2.3\%$  for arbutin. Furthermore, *B. infantis* culture filtrate significantly inhibited  $\mu$ -MSH-induced tyrosinase activity in a dose-dependent pattern (Figure 1C). After those treatments, the remained B16F10 intracellular tyrosinase activity was  $94.5 \pm 4.4$ ,  $81.04 \pm 3.5$ ,  $51.1 \pm 3.8$ ,  $21.8 \pm 2.9\%$  for 1.0, 2.5, 5.0 and 7.5% of bacterial culture filtrate, respectively. Meanwhile, the intracellular tyrosinase activity  $93.7 \pm 1.3\%$  for kojic acid and  $89.4 \pm 4.6\%$  for arbutin. Chronic exposure to solar UV radiation plays a key role in the initiation of several skin disorders, including scaling, wrinkling, dryness and mottled pigment abnormalities such as hypopigmentation and hyperpigmentation (Ichihashi et al., 2003; Mukhtar and Elmets, 1996). Therefore, there is an increasing need for new and effective agents which perform photo protection and skin whitening activities to prevent the above skin disorders. So far, there is no report about the effect of probiotic bacterial culture filtrate on melanin production. The results shown in Figure 1C were in accordance with the results indicated in Figure 1B, which means *B. infantis* culture filtrate inhibit intracellular tyrosinase activity and then decrease the melanin content in a dose-dependent manner in B16F10 melanoma cells. In the experiments,  $\mu$ -MSH was used as a cAMP inducer to stimulate melanin synthesis. It is evidenced that -MSH can bind melanocortin 1 receptor (MC1R) and activate adenylate cyclase, which in turn catalyzes ATP to cAMP and increases intracellular cAMP level (Ballotti, 2000). In this report, the results show that *B. infantis* culture filtrate inhibited melanogenesis induced by  $\mu$ -MSH mediated intracellular cAMP up-regulation. The skin is exposed to UV and environmental oxidizing pollutants and is a preferred target of oxidative stress. It is reported that UV irradiations induce the formation of ROS in cutaneous tissue provoking toxic changes such as lipid peroxidation and enzyme inactivation (Emerit, 1992). To counteract the oxidative damage, skin is equipped with a network of enzymatic and non-enzymatic antioxidant systems. To elucidate the antioxidant activity of *B. infantis* culture filtrate, DPPH, ABTS<sup>+</sup> radical scavenging activity and reducing power of the bacterial culture filtrate was determined as described previously. Figure 2A shows the DPPH radical scavenging activity of *B. infantis* culture filtrate is increased in a dose-dependent manner and the radical scavenging activity was  $56.76 \pm 1.1$ ,  $60.5 \pm 5.5$ ,  $91.7 \pm 3.0\%$  for 2.5, 5.0 and 7.5% of bacterial culture filtrate, respectively. The DPPH radical scavenging activity of vitamin C was  $43.5 \pm 1.13\%$  and that of vitamin E was  $35.9 \pm 1.74\%$ . Figure 2B depicts the ABTS<sup>+</sup> radical cation absorption capacity of various concentrations of *B. infantis* culture filtrate.

The radical scavenging activity for 2.5, 5.0 and 7.5% of bacterial culture filtrate was  $51.4 \pm 1.3$ ,  $59.9 \pm 1.5$ ,  $84.4 \pm$



**Figure 1.** Whitening effect of *B. infantis* culture filtrate. A. Mushroom tyrosinase activity assay. B. B16F10 melanin content determination. C. B16F10 intracellular tyrosinase activity measurements. Results are represented as percentages of control, and the data are presented as mean  $\pm$  S.D. of three separate experiments. Values are significantly different by comparison with the control. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 2.** Antioxidant effects of *B. infantis* culture filtrate. A. Determination of DPPH scavenging capacity. B. ABTS<sup>+</sup> radical cation scavenging activity measurement. C. Reducing power assay. All assays were performed as described in Materials and methods. Values are represented as percentage of control. Data are means  $\pm$  S.D. of three separate experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

activity of vitamin C (60  $\mu$ M) was  $97.1 \pm 0.33\%$  and that of 0.6%, respectively. Meanwhile, the radical scavenging vitamin E (60  $\mu$ M) was  $46.4 \pm 6.3\%$ . The reducing power of *B. infantis* culture filtrate increased steadily with the increasing concentration (Figure 2C). In this study, the reducing power of bacterial filtrate, vitamin C and vitamin E was compared with that of butylated hydroxyanisole (BHA) (0.03 mg/ml). The reducing power for 0.5, 1.0 and 1.5% of bacterial culture filtrate was  $22.5 \pm 1.2$ ,  $35.7 \pm 2.4$ ,  $45.8 \pm 2.3\%$ , respectively. Meanwhile, the reducing power of vitamin C (44  $\mu$ M) was  $36.5 \pm 2.5\%$  and that of vitamin E (44  $\mu$ M) was  $16.4 \pm 0.8\%$ . This is the first report about the effect of *B. infantis* culture filtrate on melanin production. In the present study, it is found that *B. infantis* culture filtrate inhibit melanin synthesis significantly in a dose-dependent pattern. Besides, *B. infantis* culture filtrate also expresses potent antioxidant activity. The results suggested that *B. infantis* culture filtrate decreased melanin production may be attributed to its inhibitory action upon the signaling pathway regulating tyrosinase activity or depletion of cellular ROS. We will study the effects of various protein kinase inhibitors on melanogenesis in *B. infantis* culture filtrate treated B16F10 melanoma cells in the near future. Certainly, we will also analyze the active components in the *B. infantis* culture filtrate and elucidate the antioxidant and whitening mechanisms.

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